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PITUITARY CONTROL OF ADAPTATION TO FRESH WATER IN THE TELEOST GENUS *FUNDULUS*

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Despite intensive investigation in recent years of the phenomenon of hypophysial control of teleostean osmoregulation in fresh water by prolactin (see reviews by Ball, 1969a, 1969b; Ensor and Ball, 1972; Lam, 1972), several aspects of this problem have remained little explored. Burden (1956) reported that hypophysectomized *Fundulus heteroclitus* cannot survive indefinitely in half strength Long Island Sound water (salinity 13‰), a medium somewhat hyperosomotic to the blood, although they can in full strength Sound water. This is difficult to reconcile with the widely-held assumption that failure of hypophysectomized fish is due solely to an inability to maintain normal internal electrolyte levels in the face of an electrolyte-poor environment. The effect of temperature on the failure of hypophysectomized fish in fresh water has been little studied, although it is well known that temperature has a profound effect on osmoregulation in fishes (Kinne, 1967). Broad comparisons of survival after hypophysectomy have been made between species (Schreibman and Kallman, 1966) despite the fact that experimental temperatures differed between many of the studies cited. Pickford, Pang, Stanley and Fleming (1966) found that high environmental calcium levels permit *Fundulus zebrinus* (= *F. kansae*, *cf.* Drewry, 1967) to survive in fresh water after hypophysectomy but had no observable effect on the failure of *F. heteroclitus*. Ball (1969b) noted that another species of this genus, *F. diaphanus*, could survive after hypophysectomy for long periods in low calcium fresh water.

Investigations on the control of osmoregulation by the pituitary in teleosts have led to speculation on the implications of this phenomenon in terms of vertebrate evolution (Bern, 1967) and more specifically in terms of teleost evolution (Schreibman and Kallman, 1966, 1969). The latter authors, studying poeciliid fishes and some of their relatives, but making broader generalizations from data published on other species, have suggested that hypophysial control of freshwater tolerance may be of limited taxonomic distribution among teleosts and that normal environmental salinity has little influence on pituitary-dependent survival in fresh water. These conclusions must be regarded with some caution, however, as it appears that survival may be a misleading index of the ionoregulatory economy of fish (Ball, 1969a), and studies on the hypophysial control of osmoregulation in teleosts are limited to far too few species for confident phylogenetic or ecological generalizations.

In light of questions surrounding some physiological aspects of hypophysial control of adaptation to dilute media and uncertainties regarding the respective contributions of environmental and phylogenetic factors to dependence on the pituitary for survival in fresh water, we have chosen to investigate the role of the pituitary

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in survival of killifishes of the genus *Fundulus* in fresh water. The genus *Fundulus*, containing some 26 mainland North American species, is ideal for such studies. It includes 9 brackish-water species with varying affinities for fresh water, 8 freshwater species with coastal distributions which may, on occasion, enter brackish water, 8 species geographically isolated in fresh water, and one inland species commonly found in saline water. In addition, the brackish water mummichog, *F. heteroclitus*, is perhaps the most thoroughly known teleost in terms of hypophysial control of adaptation to fresh water.

We have conducted a survey of 23 species and one subspecies of *Fundulus* and one species of the related Middle American genus *Profundulus* in regard to the ability to survive in fresh water after hypophysectomy. It is hoped that such an approach on a taxonomically unified but environmentally-diverse group of fishes will provide insight into the interplay between phylogenetic and ecological factors involved in the determination of the importance of the pituitary in adaptation to fresh water. In addition, we have attempted to clarify the influence which experimentally-modifiable environmental parameters (salinity, calcium, and temperature) have on failure. Such data are necessary to a rational comparison of the failure times of species whose natural environments may differ in regard to temperature range and other factors. Furthermore, we have undertaken to compare the response of blood electrolytes to hypophysectomy in different species and under different laboratory conditions in an attempt to elucidate the physiological basis for failure, and to ascertain whether it is homologous between species and within a species under different environmental conditions.

Materials and Methods

The nomenclature used in the present study generally follows that of Miller (1955). The nominal *F. kansae* and *F. similis* are regarded as subspecies of *F. zebrinus* and *F. majalis* respectively following Drewry (1967) and Relyea (1967). *F. swampinus* is recognized as a valid species following Rivas (1966, as *F. lineolatus*) and Griffith (1972). The unnamed species from Tennessee is that species referred to as the "barrens topminnow" by Miller (1972), and formerly relegated to *F. albolineatus* (Miller, 1955; Brown, 1957).

A total of 14 freshwater species of *Fundulus*, 8 species and one subspecies of brackish-water *Fundulus*, and one inland species of the genus from saline water were collected between 1968 and 1970 from their normal habitats in the eastern, southern, mid-western and Pacific coastal United States. Briefly, the brackish-water species include *F. confluentus*, *F. jenkinsi* and *F. pulvereus* from low salinity brackish environments, *F. heteroclitus* and *F. grandis* found in widely variable salinities, and *F. luciae*, *F. majalis* and *F. parvipinnis* typical of high salinities. *F. zebrinus* is an inland species found in both fresh and saline waters. Among the freshwater species, *F. cingulatus*, *F. notti* and *F. swampinus* are normally found in dilute swamp water, *F. rathbuni*, *F. stellifer* and *F. waccamensis* in fresh waters of moderate ion content, *F. catenatus*, *F. sciadicus*, *F. seminolis* and the undescribed species in fresh waters with high concentrations of dissolved minerals, and *F. chrysotus*, *F. diaphanus*, *F. notatus*, and *F. olivaceus* are widespread species which inhabit fresh waters of varied salt content. Although *F. chrysotus*, *F. cingulatus*, *F. diaphanus*, *F. notatus*, *F. notti*, *F. olivaceus*, *F. semi-*

nolis, and *F. swampinus* are all distributed in coastal regions, only *F. diaphanus* and *F. chrysotus* have been reliably reported to enter dilute brackish water environments. Further details on environmental salinities of all species are given elsewhere (Griffith, 1974).

The collection sites are generally identical to those tabulated by Griffith (1974) with the following exceptions: the *F. catenatus* in the present study included specimens from both Osage (group A) and Wright (group B) Counties, Missouri; the *F. olivaceus* were from Holmes Co., Florida (group A) and Cape Girard Co., Missouri (group B); the *F. rathbuni* were from Orange (group A) and Mecklenberg (group B) Counties, North Carolina; and the *F. swampinus* were from Columbus Co., North Carolina (group A) and Leon Co., Florida (groups B and C). Specimens of *Profundulus punctatus* were tank-raised individuals provided by Dr. Neal R. Foster (Philadelphia Academy of Natural Sciences).

For most species, young adult specimens were studied, but in the cases of *F. heteroclitus* (group B), *F. jenkinsi, F. catenatus, F. olivaceus* (group B), *F. stellifer* and *F. waccamensis* (groups B and C) juveniles were used. The groups investigated generally included both sexes although only males were studied in *F. heteroclitus* (groups A and B), *F. majalis* (group D), *F. diaphanus* (group A), *F. swampinus* (group B) and *F. seminolis,* whereas only females were tested for *F. heteroclitus* (group D) and the undescribed species.

Upon arrival in New Haven, Connecticut, freshwater species were initially adapted to running, dechlorinated New Haven tap water (salinity less than 0.1‰, calcium less than 8 ppm) and brackish or saline species were adapted to recirculated sea water (Long Island Sound water, salinity 29‰). The fish were fed a diet of modified Aronson's mixture (Pickford, 1953) liberally supplemented with frozen brine shrimp and were maintained on an 8 hour day. After laboratory acclimation of at least one month, fish were hypophysectomized by the opercular method of Pickford (1953), modified for smaller fish by cutting along the base of the operculum. Hypophysectomy of freshwater species was performed in dilute sea water (salinity 6‰), found empirically to be the medium at which recovery was optimal. Brackish-water or saline species were hypophysectomized in either sea water or dilute sea water (6, 10 or 12‰) and were permitted to recover in this medium for a period varying from a week to several months; the duration being brief in the case of dilute sea water which some brackish-water species cannot tolerate indefinitely after hypophysectomy (Burden, 1956). Post-operative mortaility prior to testing was negligible for most species.

After 8–14 days of recovery in the operation medium, freshwater species were tested by abrupt transfer to tap water. Unoperated controls were maintained continually in tap water. Hypophysectomized and unoperated brackish-water species were tested both by abrupt transfer from the operation medium to fresh water and by gradual dilution of the salinity of the home tank by allowing tap water to slowly drip in. For the latter tests, salinity declines of 1.0 to 1.8‰ per day were achieved. The symptoms of failure for both freshwater and brackish-water species were as described by Burden (1956). Loss of balance was regarded as the definitive criterion of failure. Transfer back to sea water or dilute sea water was attempted for many species after failure had occurred, and was generally successful for brackish-water species but only occasionally for freshwater species.

Completeness of hypophysectomy was judged on the bases of initial notes on the operation, failure of the fish to increase in length, abnormal fin regeneration, pallor, gonadal regression and post mortem dissection. While it is recognized that there are reports of small clusters of prolactin-secreting cells left after imperfect hypophysectomies proliferating and permitting survival in fresh water (Pickford, Robertson, and Sawyer, 1965; Ball, 1965a, 1965b; Schreibman and Kallman, 1969), and that regeneration of that portion of the pituitary involved in osmoregulation need not be accompanied by regeneration of other portions, it is most unlikely that this could be the case in the vast majority of specimens in the present study in which the pituitary was observed to be removed as an intact organ. At least one of the collateral parameters on which completeness of hypophysectomy was judged (pallor) is primarily a function of the same hormone responsible for freshwater survival in *F. heteroclitus*. In this species melanogenesis is under prolactin control while only the proliferation of new melanophores is affected by intermedin (Pickford and Kosto, 1957). Specimens for which there was any doubt regarding the completeness of hypophysectomy have not been considered in our study.

In addition to studies of survival in fresh water for all species of *Fundulus*, we have assessed the roles of environmental calcium and salinity on the failure time of *F. heteroclitus*. For the former experiment, water with a calcium concentration of 370 ppm was obtained by adding $CaCl_2$ to tap water. For the latter studies, sea water was diluted with tap water to obtain salinities of 1, 3, and 5‰ in one experiment and 1, 6, and 12‰ in a second. The effect of temperature (20°, 15°, 8°, and 1° C) was tested on the freshwater failure of *F. heteroclitus*. In this study the specimens were hypophysectomized in sea water at 15° C and were then acclimated to the test temperature for at least one month prior to testing. While most other species were tested only at 15° C, several (*F. luciae*, *F. chrysotus*, *F. olivaceus*, *F. swampinus* and *F. waccamensis*) were tested at both 15° and 20° C, and two (*F. notti* and *Profundulus punctatus*) were tested at only 20° C. 15° C was chosen as the principal experimental temperature to minimize problems of sexual activity in control fish and infection in the specimens, and also to facilitate comparison with other studies on *Fundulus*, most of which have been conducted at 15° C. All populations of *Fundulus* tested, with the possible exceptions of *F. parvipinnis* from Southern California and *F. confluentus* from peninsular Florida, were collected in geographical areas in which environmental water temperatures well below 15° C are normal during the winter.

Blood electrolyte levels were measured in two brackish-water species (*F. majalis* and *F. heteroclitus*) and in three freshwater species (*F. diaphanus*, *F. swampinus* and *F. rathbuni*). Blood was collected from intact and hypophysectomized *F. heteroclitus* in sea water, hypophysectomized fish failing in fresh water, dilute sea water on high calcium fresh water, and mock-operated controls sacrificed after 12 days in fresh water or high calcium fresh water. Intact and hypophysectomized *F. majalis* were studied in sea water and failing in fresh water. Blood electrolyte studies on the three freshwater species were conducted on fish hypophysectomized in dilute sea water (6‰), transferred to fresh water 8–14 days after the operation, and sacrificed after a period of time in this medium (29 days for *F. rathbuni*, 31 days for *F. swampinus*, and at 8 and 48 days for *F. diaphanus*). Blood from specimens failing in fresh water was also collected for *F. rathbuni* and *F. diaphanus*, and blood of intact and hypophysectomized *F. diaphanus* was collected after one week in sea water following a very gradual increase in salinity. For the blood studies, free-flowing blood was collected in non-heparinized microhematocrit tubes from the severed caudal penuncle (or by heart puncture in the case of *F. swampinus*), permitted to clot, centrifuged, and 5 μl aliquots of serum were used for the determination of chloride (Amino-Cotlove chloride titrator) and sodium and potassium (Instrumentation Laboratory flame photometer) following procedures described in Pickford, Grant and Umminger (1969).

TABLE I

Effect of temperature, salinity and calcium on the survival of hypophysectomized F. heteroclitus

Group	Pre-transfer medium		Experimental medium		N	Hours until failure	
	Salinity	Days adapted	Salinity	Temperature		Mean ± S.E.	Range
A1	29‰	105	FW	15°	3	129 ± 18	94–152
B1	29‰	48	FW	20°	7	79 ± 22	10–117
B2	29‰	48	FW	15°	7	86 ± 21	19–166
B3	29‰	48	FW	8°	9	232 ± 35*	126–476
B4	29‰	48	FW	1°	9	251 ± 45*	117–476
B5	29‰	30	1‰	20°	7	137 ± 16	68–187
B6	29‰	30	3‰	20°	6	359 ± 9†	334–388
B7	29‰	30	5‰	20°	8	246±46†	101–428
C3	29‰	30	FW	20°	7	146 ± 21	96–264
C5	29‰	30	1‰	20°	6	451 ± 73††	194–622
C6	29‰	30	6‰	20°	5	504 ± 164††	264–1152
C7	29‰	30	12‰	20°	6	787 ± 209††	271–1326
C4	29‰	30	FW + Ca^{++}	20°	6	166 ± 17	125–228

* Significantly different from both 15° and 20°, $P < 0.01$.
† Significantly different from appropriate freshwater controls (group B1), $P < 0.01$.
†† Significantly different from freshwater controls, $P < 0.01$.

The statistics presented in this paper are means ± standard errors calculated for small sample numbers and statistical significance, when appropriate, was determined using Student's *t* test. For several species, difficulties in collection or transportation prevented the study of adequate numbers of specimens for proper statistical analysis. Such data have been included for the sake of completeness, although it is recognized that small numbers may preclude very strong interpretations.

RESULTS

Effect of temperature on survival in fresh water after hypophysectomy

Data is presented in Table I demonstrating the effect which adaptation temperature has on failure time in hypophysectomized *Fundulus heteroclitus* in fresh water. In this species low temperatures retard failure. Survival time at high temperatures (groups B1 at 20° and B2 at 15° C) is roughly one-third that at low temperatures (groups B3 at 8° and B4 at 1° C). There is little difference in any of the experiments between failure time at 15° and 20° C in *F. heteroclitus*, and there also appears to be little difference between 8° and 1° C; a discontinuity between long and short survival seems to occur between 15° and 8° C in this species. We might note that while we found no significant difference in time until failure between 15° and 20° C, a major difference was found in successful recoveries of failing fish returned to sea water. At 20° C only 29% of the failing fish could be saved. At 15°, 8°, and 1° C the respective survivals were 71%, 89%, and 77%. It would appear that the progression of osmoregulatory failure after the first symptoms appear is more rapid at the higher temperature.

TABLE II

Comparison of survival of brackish-water species of Fundulus *in fresh water after hypophysectomy*

Species	Group	Pre-transfer medium		Temperature	Number	Hours until failure	
		Salinity	Days adapted			Mean ± S.E.	Range
F. confluentus	A1	29‰	12	15°	1	57	
	A2	10‰	10	15°	1	106	
F. grandis	A1	29‰	17	15°	3	137 ± 39	94–215
F. jenkinsi	A1	10‰	10	15°	4	200 ± 20	150–240
	A2	29‰	9	15°	4	197 ± 45	148–330
F. luciae	A1*	6‰	14	20°	7	23 ± 0	23
	A2*	6‰	16	15°	5	35 ± 5	25–47
	A3*	12‰	5	15°	2	43	43
F. majalis majalis	B1*	29‰	64	15°	8	18 ± 7	9–70
	C1*	12‰	1	15°	5	24 ± 4	21–30
	D1*	6‰	7	15°	3	57 ± 21	25–97
F. majalis similis	A1	10‰	13	15°	2	111	94–127
	A2	29‰	10	15°	1	45	
F. pulvereus	A1	10‰	10	15°	3	139 ± 20	92–153
	A2	29‰	9	15°	2	126	103–148
F. zebrinus	A1	FW + Ca^{++}	30	15°	2	372	144–600

* Some failure observed in intact controls in fresh water. See text for details.

A number of species in addition to *F. heteroclitus* were tested at both 15° and 20° C. In the brackish-water species, *F. luciae,* high temperature accelerates failure in both intact and hypophysectomized fish. Under conditions of rapid transfer from dilute saline (6‰) to fresh water, the intacts fail within 5–8 days at 20° and survive for over a month at 15° C, while hypophysectomized fish fail within one day at 20° and between one and two days at the lower temperature (Table II). Among the freshwater species there appears to be some acceleration of failure at 20° in *F. olivaceus, F. swampinus* and *F. waccamensis,* while in *F. chrysotus* there is, if anything, longer survival at the higher temperature (Fig. 1). There is no apparent correlation of the effect of temperature on freshwater failure with geographic latitude or with environmental salinity. The opposing data on the freshwater species *F. chrysotus* and *F. swampinus* were from fish collected within a few miles of each other and the same is true of the data on the brackish-water *F. heteroclitus* and *F. luciae* tested at 15° and 20° C.

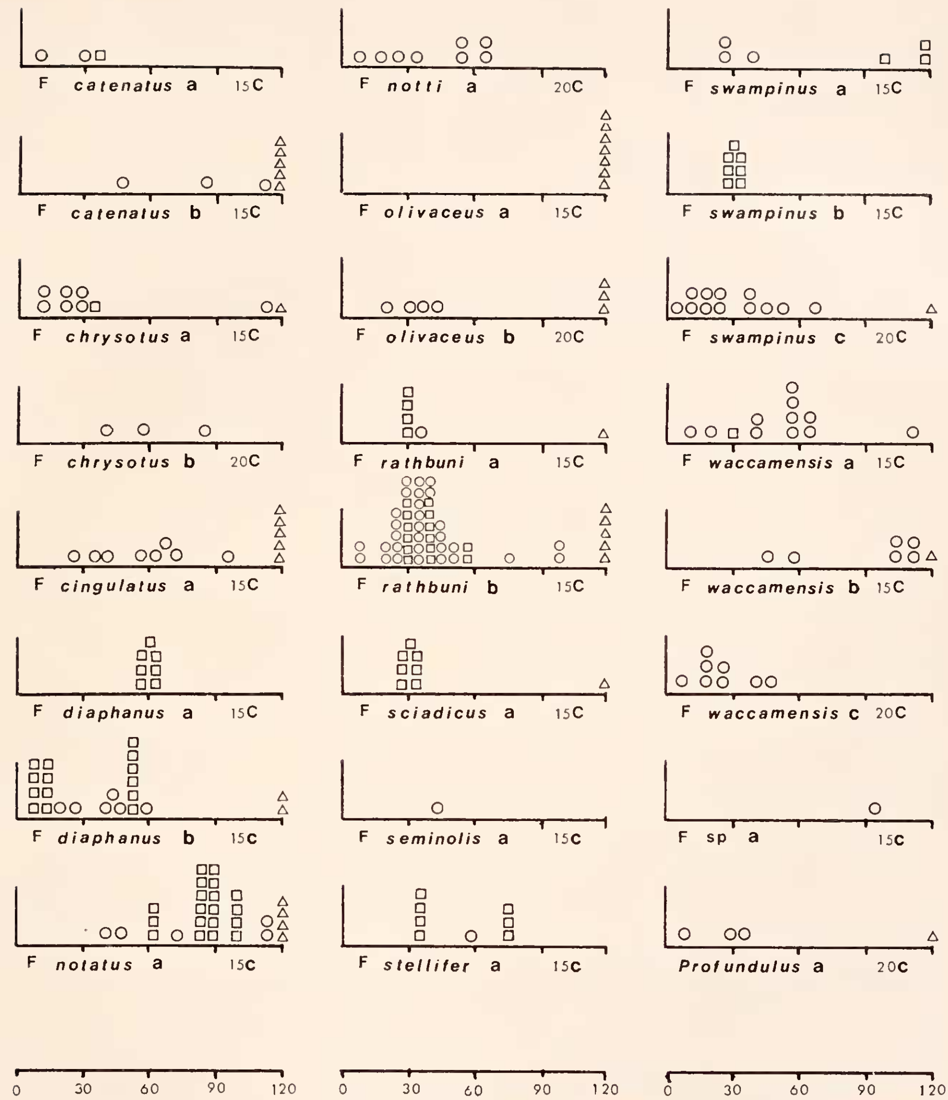

FIGURE 1. Survival of hypophysectomized freshwater species of *Fundulus* and *Profundulus* in fresh water. The number of days since transfer to fresh water (horizontal axis) is given at the bottom. Failing fish are depicted as circles, fish removed from the experiment as squares, and fish surviving over 120 days as triangles. Group designation and experimental temperature are shown for each species.

A consideration of published reports on the failure of *F. heteroclitus* indicates that studies conducted at 15° (Burden, 1956; Pickford, Robertson and Sawyer, 1965) give results in agreement with ours, while Potts and Evans (1966) reported failure times of less than one day at 20° C, in contrast to our data which indicate failure time at this temperature of 3–6 days. Potts and Evans adapted their fish to 40% sea water, a medium in which hypophysectomized *F. heteroclitus* cannot survive indefinitely (Burden, 1956), and it is possible that the fish studied by these authors were already near failure at the time they were transferred to fresh water. Our demonstration that low temperature generally prolongs survival after hypophysectomy of species of *Fundulus* in fresh water is in accord with the results of Chidambaram, Meyer and Hasler (1972) on *Ictalurus melas*.

Effects of environmental salinity and calcium on survival of F. heteroclitus

Table I presents data on the influence of salinity (compare groups B1, B5, B6, and B7 for experiment one and C3–C7 for the second) and calcium (groups C3 and C4) on the survival of hypophysectomized *F. heteroclitus* at 20° C. In both salinity experiments the addition of sea water to the tank significantly prolonged survival over that of fish in regular tap water. In the second experiment a progressive increase in survival time was found with increasing salinity although this was not clear in the first experiment. Failure was even observed at 12‰, a salinity at which the calculated osmolality (366 mOsm/kg) is essentially identical to serum osmolality of intact *F. heteroclitus* in sea water (Pickford *et al.*, 1969). This observation confirms the studies of Burden (1956) who reported that hypophysectomized *F. heteroclitus* fail at 13‰, and of Pickford *et al.* (1965) who found that the failure of hypophysectomized *F. heteroclitus* subjected to gradual decreases in salinity was indicative of an initiation of the process at salinities near those observed by Burden (1956) to lead to eventual failure. We should note here that the somewhat shorter survival observed for the groups in the first experiment may be attributable to size differences. In the first investigation very small juveniles (1–2g) were used, while for other experiments on *F. heteroclitus* young adults (generally over 4g) were studied.

We failed to find any prolongation of survival of hypophysectomized *F. heteroclitus* when high levels of calcium (370 ppm Ca^{++} as $CaCl_2$) were added to tap water. The fact that in the same experiment survival was significantly prolonged at 1‰, a salinity at which chloride levels are lower and osmolality only slightly higher than the calcium-rich tap water, suggests that the beneficial effect of dilute sea water on survival of hypophysectomized *F. heteroclitus* is likely due to sodium concentration. Our negative findings on the effect of calcium on *F. heteroclitus* confirm the data of Pickford, Pang, Stanley and Fleming 1966 on this species, and contrast with the positive results of the same authors on *F. zebrinus*.

Comparison of survival of hypophysectomized Fundulus *species and* Profundulus *in fresh water*

Brackish-water species of *Fundulus* tested by rapid transfer from sea or dilute sea water to fresh water demonstrate mean times until failure ranging from about one day in *F. majalis* and *F. luciae* to eight days in *F. jenkinsi* (Table II). Data on survival of intact control specimens has been presented elsewhere (Griffith, 1974) and, because failure of such specimens was the exception, are not repeated in the tables. When failure did occur it is noted in the text. Specimens of *F. zebrinus,* an inland species frequently found in saline water, fail after about two weeks when transferred from high calcium fresh water to normal tap water. The remaining brackish-water species show mean survival of 2–5 days at 15° C. There appears to be no consistent effect of the pre-adaptation medium on failure in the species tested. In *F. majalis* a period in dilute sea water has an ameliorative effect, but this appears not to be the case with some of the other species. Failure of intact *F. luciae* was observed at 20° but not at 15°. *F. majalis* intacts failed at the same time as hypophysectomized fish when subjected to abrupt transfer from sea to fresh water but survived well when permitted to adapt to dilute sea water (6‰) prior to testing. With the exception of *F. parvipinnis*, intacts of which fail within a day of transfer to fresh water from sea water but hypophysectomized specimens

TABLE III

Salinity at failure for hypophysectomized brackish-water species of Fundulus *subjected to a gradual decrease in salinity at 15° C. Values in brackets exclude a single abnormally high fish.*

Species	Group	Number	Rate of decrease in ‰/day	Salinity at failure (‰)	
				Mean ± S.E.	Range
F. zebrinus	A	6	1.1	0.95 ± 0.65	0.1–4.2
				[0.30 ± 0.09]	[0.1–0.5]
F. jenkinsi	A	3	1.4	3.20 ± 0.35	0.3–8.9
				[0.35]	[0.3–0.4]
F. grandis	A	8	1.1	1.33 ± 0.05†	1.1–1.5
F. confluentus	A	5	1.2	1.54 ± 0.14	1.3–1.9
F. heteroclitus	A	5	1.0	1.64 ± 0.07	1.2–2.0
F. luciae	A	8	1.3	3.44 ± 1.22*	0.4–18.0
				[1.36 ± 0.33]	[0.4–3.3]
F. majalis	A	3	1.3	3.10 ± 0.35*	2.6–3.8
F. m. similis	A	3	1.2	3.22 ± 1.23	0.9–5.0
F. pulvereus	A	3	1.4	3.40 ± 0.10	3.2–3.5
F. parvipinnis	A	1	1.8	4.20**	

† One (of 8) intact controls failed at a salinity of 1.3‰.

* Some failure occurred in intact controls after several months in fresh water.

** Of 3 intact controls, one failed at 4.1‰ and the others shortly after the salinity fell below 1.0‰.

were not tested by this procedure, failure was not observed in intact controls of the other species.

Hypophysectomized brackish-water species tested by gradual dilution of their milieu failed at salinities between about 1‰ to 4‰ (Table III). Considerable variability in the salinity at failure was in evidence both within and between species. The former is especially true of *F. jenkinsi*, *F. luciae* and *F. zebrinus* where the omission of a single abnormally high value would substantially reduce the mean. We are hesitant to aribitarily exclude these values as failure in *F. heteroclitus* can occur in media iso- or even somewhat hyperosmotic to the body fluids (Burden, 1956; this report). With the exception of *F. parvipinnis*, intact controls generally survived well when subjected to gradually-decreased salinity. One (of 3) intact *F. parvipinnis* failed at 4.1‰ and the others failed shortly after the salinity fell below 1.0‰. A single (of 8) intact *F. grandis* failed at 1.3‰, and some delayed failure was observed in intact *F. majalis* and *F. luciae* after several months in fresh water. It is worth noting that when allowance is given for the time until failure following abrupt transfer and the additional delay in failure time in dilute sea water, failure tested by gradual dilution is consistent with an initiation of the process at salinities near presumed iso-osmotic levels for most brackish-water species.

Of the 14 freshwater species of *Fundulus* and one species of *Profundulus* in which freshwater tolerance was tested, none showed average times until failure of less than one month (Figure 1). Practical considerations prevented the continuation of most tests past a few months, but in the cases where this was possible (*F. rathbuni*, *F. notatus*, *F. olivaceus*, and *F. cingulatus*) the pattern of failure showed a gradual attrition as is also suggested by abbreviated tests on most other species. Even in specimens failing after very long periods of time (over one year in the case of some *F. notatus*) the symptoms accompanying failure (asthenia, extreme pallor, loss of balance) were identical to those in failing brackish-water species. The studies on *F. rathbuni* are indicative of high mortality occurring at about one month after transfer to fresh water. In this species a very high percentage (72%) of hypophysectomized specimens develop kidney stones after a few weeks which can occlude the kidney ducts and might be responsible for failure (Griffith and Pang, 1969). Such stones may also occur in a substantial percentage of hypophysectomized *F. heteroclitus* (Pickford, 1953), *F. diaphanus*, *F. waccamensis*, *F. catenatus*, *F. olivaceus* and in a few specimens of some other species. The relatively short survival of hypophysectomized *F. notti* and *Profundulus punctatus*, which were tested only at 20° C, might be extended somewhat for comparison with species tested only at 15° since survival at 20° appears to be shorter in most species of *Fundulus* tested at both temperatures.

A clear dichotomy exists between those species of *Fundulus* characteristic of brackish-water and those characteristics of freshwater environments in regard to survival in fresh water after hypophysectomy. The former fail within about a week or less and the latter survive for at least one month and generally much longer. *F. zebrinus*, an inland species which may frequent either freshwater or saline environments (Lewis, 1957) shows an intermediate condition. In this species environmental calcium plays a very important role in adapting to fresh water (Pickford, Pang, Stanley and Fleming, 1966). Within the brackish-water species the correlation between normal environmental salinity and survival in fresh water after hypophysectomy is still in evidence. Abbreviated survival is found in *F. parvipinnis*, *F. luciae* and *F. majalis*, the most halophilic species in nature (Griffith, 1974), while *F. jenkinsi* which survives the longest is found at low salinities. The relationship breaks down within the freshwater species. While freshwater species of *Fundulus* inhabit a variety of different environments including swamp water with very low levels of dissolved electrolytes (*F. cingulatus*, *F. swampinus*, *F. notti*), mineral-rich streams or lakes (*F. catenatus*, *F. sciadicus*, *F. seminolis*, *F.* sp.), intermediate conditions (*F. waccamensis*, *F. rathbuni*, *F. stellifer*), or a variety of different habitats (*F. chrysotus*, *F. notatus*, *F. olivaceus*, *F. diaphanus*) there is not even a suggestion that these habitat differences are reflected in survival time. *F. diaphanus*, which not infrequently enters low brackish water in estuaries, shows survival times very similar to those of its close relative *F. waccamensis*, a lake endemic.

There is little evidence that differences between species of *Fundulus* in freshwater tolerance after hypophysectomy are strongly correlated with evolutionary relationships within the genus. *F. notatus* and *F. olivaceus*, a closely related species pair, share very long survival times. On the other hand, what differences there are between survival in the other freshwater species are not suggestive of evolutionary stability of this feature. *F. catenatus*, *F. stellifer* and *F. rathbuni* form a close group phylogenetically, but *F. rathbuni* fails relatively rapidly and the others slowly. Nor is there any evidence for a close correlation between pituitary-dependent freshwater tolerance and various phylogenetic schemes proposd for the brackish-water species of *Fundulus* (*cf.* Brown, 1957; Miller, 1955; Farris, 1968; Chen; 1971; Relyea, 1967; Griffith, 1972). Since it is likely that freshwater species of *Fundulus* were derived from brackish-water ancestors by at least two independent invasions of fresh water (Griffith, 1972), and that freshwater adaptation in the genus *Profundulus* was probably independent of that in *Fundulus*, it would appear that the long survival of freshwater species of the genera *Fundulus* and

TABLE IV

Effect of hypophysectomy on serum electrolytes of F. heteroclitus *in various media at 20° C*

Group	Condition	Salinity	Time in medium	Health	Number	Serum electrolytes (values in mEq/l ± S.E.)		
						Sodium	Potassium	Chloride
D1	Intact	SW	Long-term	Good	5	181.0 ± 3.8	7.8 ± 0.2	142.1 ± 3.2
D2	Hypect.	SW	Long-term	Good	5	175.3 ± 1.2	5.9 ± 0.3	124.2 ± 5.2*
C1	Mock-op.	FW	12 days	Good	5	179.2 ± 2.4	5.8 ± 0.3	124.8 ± 1.6
C2	Hypect.	FW	4–11 days	Failing	6	67.5 ± 3.6*	17.9 ± 4.9*	30.7 ± 3.3*
C3	Hypect.	1‰	8–16 days	Failing	3	103.3 ± 10.4†	15.0 ± 4.2	31.3 ± 3.7
C4	Hypect.	6‰	11–16 days	Failing	3	132.3 ± 12.7†	20.4 ± 6.9	58.3 ± 2.7†
C5	Hypect.	12‰	24–58 days	Failing	3	155.9 ± 13.1†	26.1 ± 6.3	138.0 ± 29.1†
C6	Mock-op.	FW + Ca^{++}	12 days	Good	4	183.0 ± 3.5	7.8 ± 0.9	132.8 ± 2.1†
C7	Hypect.	FW + Ca^{++}	5–9 days	Failing	6	77.4 ± 4.0*	11.8 ± 3.3	37.4 ± 2.8*

* Significantly different from intact or mock-operated controls in same medium, $P < 0.05$.
† Significantly different from comparable freshwater group, $P < 0.05$.

Profundulus is principally determined by the environmental history of the species and that stability of the feature within phylogenetic lines plays a subsidiary role.

Because of the intolerance of most freshwater species of *Fundulus* for sea water (Griffith, 1974) and the poor survival of some brackish-water species in fresh water, it was generally not possible to use identical experimental methods on the two groups of fishes. Hence, we must at least consider the possibility that differences in method might account for the observed differences between the two groups in respect to freshwater tolerance after hypophysectomy. This would appear to be most unlikely from the fact that brackish-water species fail even when environmental salinity is reduced very gradually or when they are hypophysectomized in dilute sea water and then tested by transfer to fresh water as were the freshwater species. Burden (1956) tested *F. heteroclitus* hypophysectomized in fresh water and found little difference in failure time between such fish and those hypophysectomized in sea water and tested by abrupt transfer to fresh water. Mean failure time was 7.7 days for the former and 6.5 days for the latter, although a few freshwater-adapted fish held on for over two weeks.

Effect of hypophysectomy on serum electrolytes in five species of Fundulus

Data is given in Tables IV and V demonstrating the effect of hypophysectomy on serum electrolytes (sodium, chloride and potassium) in five species of *Fundulus.* In *F. heteroclitus* (Table IV), hyphophysectomy has only minor effects on blood electrolytes of seawater fish but results in profound changes following transfer to fresh water. In sea water (groups D1 and D2), there is a barely significant ($0.02 < P < 0.05$) depression of serum chloride but no change in serum sodium or potassium. Blood taken at the time of failure from hyphophysectomized fish in fresh water (group C2) showed a 75% decline in chloride, 62% decline in sodium and a 200% increase in potassium when compared with serum of mock-operated controls killed after 12 days in fresh water (group C1). A comparison of our data with that of Pickford, Griffith, Torretti, Hendler and Epstein (1970) on

TABLE V

Effect of hypophysectomy on serum electrolytes of four species of Fundulus *in fresh or sea water (15° C)*

Species	Group	Condition	Salinity	Time in medium	Health	Number	Serum electrolytes (values in mEq/l ± S.E.)		
							Sodium	Potassium	Chloride
F. majalis	E1	Intact	SW	Long-term	Good	7	175.1 ± 2.9	11.3 ± 1.8	163.0 ± 2.4
	E2	Hypect.	SW	Long-term	Good	5	167.8 ± 5.5	12.6 ± 3.0	155.0 ± 10.4
	C1	Intact	FW	8–49 hours	Failing	5	92.6 ± 5.1	20.7 ± 4.6	63.3 ± 3.9
	C2	Hypect.	FW	21–30 hours	Failing	5	76.4 ± 4.6*	35.3 ± 4.3*	49.1 ± 5.1*
F. diaphanus	A1	Intact	SW	7 days	Good	7	189.3 ± 7.0	5.3 ± 0.8	148.4 ± 6.7
	A2	Hypect.	SW	7 days	Good	5	184.1 ± 5.0	9.6 ± 3.1	168.1 ± 2.7*
	B1	Intact	FW	Long-term	Good	6	143.0 ± 1.5	9.3 ± 0.4	106.7 ± 6.3
	B2	Hypect.	FW	8 days	Good	8	136.4 ± 2.0*	11.9 ± 1.3	104.8 ± 3.3
	B3	Hypect.	FW	48 days	Good	6	111.6 ± 3.6**	18.6 ± 1.8	63.0 ± 2.8**
	B4	Hypect.	FW	16–48 days	Failing	6	75.1 ± 3.8**	24.7 ± 0.7*	39.8 ± 2.0**
F. rathbuni	B1	Intact	FW	Long-term	Good	4†	—	—	112.4 ± 1.2
	B2	Hypect.	FW	29 days	Good	6	—	—	84.1 ± 3.1*
	B3	Hypect.	FW	12–200 days	Failing	11	—	—	58.8 ± 5.3**
F. swampinus	B1	Intact	FW	Long-term	Good	5	—	—	97.8 ± 3.4
	B2	Hypect.	FW	31 days	Good	7	—	—	88.9 ± 4.1

* Significantly different from intact controls, $P < 0.05$.
** Significantly different from both intact controls and preceding hypect. group, $P < 0.05$.
† Pooled samples, two fish per sample.

hypophysectomized fish autopsied after 3 days in fresh water, indicates that such fish have serum chloride and sodium levels intermediate between those of intact fish in fresh water and failing hypophysectomized fish in this medium. This suggests that the decline in serum electrolytes in *F. heteroclitus* following hypophysectomy in fresh water is a gradual process, although it must be noted that the study of Pickford *et al.* (1970) was conducted at 15° C and the data are not directly comparable with ours, which were obtained from 20° C fish.

In *F. majalis* (Table V) we found no significant effect of hypophysectomy on blood electrolytes in sea water, although there is a trend towards slightly lower sodium and chloride levels. Both intact and hypophysectomized *F. majalis* are unable to tolerate abrupt transfer from sea to fresh water and fail at the same time (22.4 ± 7.5 hours for the intacts and 23.9 ± 1.6 hours for hypophysectomized fish). Nevertheless, intacts at failure have significantly higher serum sodium and chloride and significantly lower potassium than comparable hypophysectomized fish.

Hypophysectomized *F. diaphanus* in sea water have barely significantly higher serum chloride than intacts but other blood electrolytes are close to control levels. In fresh water there clearly are progressive declines with time of serum chloride and sodium and a concomitant increase in potassium in hypophysectomized fish. At the time of failure serum chloride values are 38%, sodium levels 52%, and potassium levels 265% the values of intact controls in fresh water.

Only serum chloride levels were measured for *F. rathbuni* and *F. swampinus*. Both species survive for fairly long periods in fresh water following hypophysectomy. In *F. rathbuni* sacrificed after 29 days in fresh water, hypophysectomized fish show significantly lower serum chloride when compared with intact controls. Serum chloride is further depressed in hypophysectomized fish showing the symptoms of failure. In *F. swampinus* there is a suggestion of lower chloride levels in hypophysectomized fish sampled after 31 days in fresh water, although the difference is not significant ($0.1 < P < 0.2$).

Several features in common, as well as a number of differences, are evident when a comparison is made of the responses of the species of *Fundulus* to hypophysectomy In the three species studied, serum electrolyte changes after hypophysectomy in sea water are minor, involving slight decreases in serum chloride in *F. heteroclitus* and slight increases in *F. diaphanus*. Changes after hypophysectomy in seawater-adapted *F. heteroclitus* were documented in much more detail by Srivastava and Pickford (1972) who found significant decreases in serum sodium and chloride as well as changes in many other blood constituents. Our data confirm the chloride data and we also note a trend towards lower sodium in *F. heteroclitus* and similar trends for both sodium and chloride in *F. majalis*. The fact that *F. diaphanus* exhibits the opposite response with chloride rising rather than falling after hypophysectomy, may reflect species differences influenced by habitat between this normally freshwater species and the brackish-water *F. heteroclitus* and *F. majalis*.

In fresh water it is clear that hypophysectomy results in profound changes in blood electrolytes in at least four of the five species tested which evenutally lead to extremely low values for serum sodium and chloride and high potassium. With the possible exception of *F. rathbuni*, the levels of serum ions at the time of failure are not significantly different between species, suggesting that tissue tolerance to lowered blood electrolyte levels is essentially identical in *F. majalis, F. heteroclitus* and *F. diaphanus*. Some failing *F. rathbuni* have relatively high serum chloride levels (up to 78 mEq/l), while others are in the "normal" range for failing fish of the other species (31–38 mEq/l). It is possible that the former were failing from causes other than depressed blood electrolyte levels. It is worth noting that the levels of serum chloride we find in failing *Fundulus* species are lower than those reported by Burden (1956), but that levels of both sodium and chloride are similar to those reported to be consistent with failure in the goldfish, *Carassius auratus*, by Lahlou and Sawyer (1969). It is possible that Burden sampled fish earlier in failure than we did. The quantitatively smaller depression of serum osmolality reported by Pickford, Pang and Sawyer (1966) probably reflects increases in serum constituents such as potassium in failing fish.

In *F. diaphanus* our data show that the decline in serum sodium and chloride are progressive and this is also strongly suggested by the data on *F. rathbuni* and *F. heteroclitus*. We have no reason to suspect this not to be the case in other species of *Fundulus*, and it seems reasonable that differences between species in tolerance of fresh water after hypophysectomy are the result of differences in the rate of decline of serum electrolytes. In *F. majalis* the decline in serum electrolytes is very rapid following transfer to fresh water and results in levels low enough to account for mortality within one day. The failure of intact as well as hypophysectomized fish suggests that hypophysial control (which is present as can be seen from the significant difference in serum electrolytes in failing fish and the ability of intacts to survive in fresh water following gradual acclimation) is inadequate to compensate for the rapid decline in electrolytes following abrupt transfer to fresh water. In *F. heteroclitus* the decline is somewhat slower and levels comparable to those of failing *F. majalis* do not occur until several days after transfer. Declines in serum electrolytes are yet more retarded in the freshwater species and levels low enough to be considered responsible for failure do not normally occur until a month or more after transfer from dilute sea water to fresh water.

While it is probably a fair assumption that the declines in serum electrolytes observed here are the result of imbalances between the influx of ions across the gills and the renal and extrarenal losses of electrolytes, it has recently been suggested (Duff and Fleming, 1972a, 1972b) that modifications in "sodium space" (*i.e.*, a redistribution of sodium to other body compartments) can provide a mechanism by which some species of *Fundulus* may regulate serum sodium at relatively constant levels. In contrast, Lahlou and Sawyer (1969) have reported that hypophysectomized goldfish, *Carassius auratus*, show marked declines in serum electrolytes while 'sodium space' increases, resulting in only minor changes in the pool of exchangeable sodium. Although our data do not permit us to distinguish whether differences in survival after hypophysectomy in fresh water between brackish-water and freshwater species of *Fundulus* are attributable to sodium flux rates or the capacity to modify "sodium space," the more pertinent observation is that, whatever their cause, these differences exist.

Effects of salinity and calcium on serum electrolytes of failing hypophysectomized F. heteroclitus

Data is presented in Table IV demonstrating the effect which elevated environmental salinity and calcium levels have on serum electrolytes of failing hypophysectomized *Fundulus heteroclitus*. With an increase in salinity there is a parallel increase in serum chloride and sodium of failing fish (Groups C2–C5). Specimens failing at 12‰ have serum chloride and sodium levels approaching those of intact specimens in fresh water or healthy hypophysectomized fish in sea water. It would appear that under the exceptional conditions of failure in moderately high salinities, the serum electrolytes measured are not closely related to failure. If lowered serum chloride and sodium are not causing failure in dilute sea water, it is worth considering what factors might be responsible. One possibility, suggested by the fact that high sodium levels in fish failing in dilute sea water are accompanied by very high potassium levels, is that the ratio of potassium to sodium is the critical factor. However, a comparison of sodium and potassium levels for individual failing fish indicated that this was unlikely. The correlation coefficient for sodium and potassium levels of all failing *F. heteroclitus* was 0.178 ($0.4 < P < 0.5$). A second possibility is that failure in both fresh water and dilute sea water and low serum electrolyte levels in fresh water are all consequences of a common phenomenon; perhaps a disturbance in acid-base metabolism. Of special interest in this regard is the observation of Srivastava and Pickford (1972) that hypophysectomy results in elevated bicarbonate levels in seawater-adapted *F. heteroclitus* and the evidence, summarized by Maren (1967) and Maetz (1971), that sodium and chloride uptake and acid-base metabolism share a common pathway through the carbonic anhydrase system.

High levels of environmental calcium are without effect on the serum chloride, sodium and potassum levels of failing hypophysectomized *F. heteroclitus* in fresh water (groups C2 and C7, Table IV). High environmental calcium levels enable many marine fishes to adapt to fresh water (Breder, 1934; Hulet, Massay, Joorey and Wehr, 1967), reduce chloride loss in *Gasterosteus aculeatus* (Heuts, 1944), and decrease water and sodium fluxes in *Fundulus zebrinus* (Potts and Fleming, 1970, 1971). Calcium deprivation is without effect on serum sodium, chloride or potassium in hypophysectomized *F. heteroclitus* in sea water (Pang, Griffith and Pickford, 1971) or hypophysectomized *F. diaphanus* in fresh water (Pang, Griffith and Schreibman, 1973), although such fish demonstrate marked declines in serum calcium and exhibit tetanic seizures.

Discussion

Our findings point to the likelihood that failure in fresh water after hypophysectomy is qualitatively comparable between different species of *Fundulus*. In all species tested, the symptoms of failure (extreme pallor, sluggishness, loss of balance and evenutal death) are identical. With the notable exception of the failure of *F. heteroclitus* in dilute sea water, failure in both freshwater and brackish-water species is closely correlated with very low serum sodium and chloride. The levels of these ions accompanying failure appear to be similar for both groups of species.

In contrast to the qualitative similarities, there are marked quantitative differences between species of *Fundulus* in freshwater failure after hypophysectomy which appear to be related to normal environmental salinity. The exact mechanisms by which these quantitative differences arise (*i.e.,* by reduced renal and/or extrarenal electrolyte efflux, by involvement of environmental calcium in electrolyte and water balance, by increased ion uptake, or by modification of electrolyte "spaces" in the late-failing freshwater species) have not yet been clarified and represent a rich source of problems for future investigation. However, what seems to us more pertinent here than the nature of the mechanisms by which freshwater species of *Fundulus* achieve longer survival after hypophysectomy than their brackish-water congeners is the very fact that, at least from the quantitative index of survival time, in these freshwater species the role of the pituitary is of less importance in adapting to fresh water.

It is our contention that a strong reliance on endocrine control of osmoregulation, at least in *Fundulus*, is most critical in euryhaline species which are subject in nature to variations in salinity from freshwater to marine (or higher) levels.

The principal known actions of prolactin on osmotic and ionic regulation (*cf.* reviews by Lam, 1972; Ensor and Ball, 1972) are all mechanisms by which the osmoregulatory physiology of a marine teleost can be transformed into that of a freshwater teleost (*cf.* Smith, 1930). It might be expected that strictly freshwater species, lacking any need for mechanisms of regulating ion and water balance in a hypertonic medium, would eliminate any such vestiges of marine life through evolutionary rather than through endocrine pathways, so that a metabolically-costly continuous secretion of hormones would not be necessary to survival. Euryhaline species would need both the capability of osmoregulating like a marine teleost in saline environments and an effective means, presumably hormonal, or modifying the mechanisms involved for adaptation to fresh water. While there are no stenohaline marine species of *Fundulus*, our data on the more halophilic species such as *F. majalis* suggest, and it is intuitively reasonable, that forms which have minimal exposure in nature to dilute environments neither need, nor possess, well-developed hypophysial control of osmoregulation.

Of considerable interest is the question of whether our data on *Fundulus* indicative of a close relationship between environment and the importance of the pituitary in osmoregulation, are representative of a widespread phenomenon in fishes or are only of restricted applicability to *Fundulus*. The only other series of studies of sufficient taxonomic breadth to be helpful in deciding this point are those of Schreibman and Kallman (1966, 1969) on poeciliid fishes. These authors found that species of the exclusively freshwater genus *Xiphophorus* demonstrate relatively long survival while the genus *Poecilia*, which includes a number of brackish-water species, has short survival; the shortest survival being found in the highly euryhaline *P. latipinna* and *P. formosa*. However, Schreibman and Kallman (1969) reported that *Gambusia affinis*, presumed to be ecologically similar to *Poecilia latipinna* on the basis of a report by Krumholz (1948), has relatively long survival and concluded that there was no consistent relationship between environmental salinity and survival after hypophysectomy in fresh water. An examination of more recent extensive field surveys (Kilby, 1955; Simpson and Gunter, 1956; Renfro, 1960; Tagatz and Dudley, 1961; Tagatz, 1968) reveals, however, that *G. affinis* is normally an inhabitant of fresh water and only sporadically enters low brackish environments, while *P. latipinna* is fully euryhaline in nature. Thus, the poeciliid data of Schreibman and Kallman (1966; 1969) exhibit a rather good correlation between environmental salinity and freshwater survival after hypophysectomy.

Generalizations for teleosts as a whole are complicated both by the scattered nature of the available data on most groups and the widely varying evolutionary and environmental history of different teleost taxa. Schreibman and Kallman (1966; 1969), in summarizing the studies on teleosts, concluded that certain taxonomic groups (*i.e.*, the ostariophysian, anguillid and perhaps salmonid fishes) appeared not to require the pituitary for survival in fresh water while the atheriniform fishes (which include *Fundulus*, the poeciliids, and a number of other groups, only a few of which have been tested) did. These authors further suggested that within teleosts as a whole there was no consistent relationship between environmental salinity and pituitary-dependent freshwater tolerance. Some doubt as to the validity of the suggested taxonomic restriction of the hypophysial mechanism for adapting to fresh water is raised by observations that some ostariophysian and salmoniform fishes require the pituitary to survive in fresh water (Chidambaram *et al.*, 1972; Stanley and O'Connell, 1970), other ostariophysian and some anguillid fishes need the pituitary for maintenance of serum electrolytes (Ogawa, 1968; Lahlou and Sawyer, 1969; Donaldson, Yamazaki and Clarke, 1968; Olivereau and Chartier-Baraduc, 1966; Chan, Chester Jones and Mosley, 1968; Butler, 1967), and our data on *Fundulus* (an atheriniform genus) represent both the shortest (about one day in *F. luciae* and *F. majalis*) and longest (up to a year or more in some *F. olivaceus* and *F. notatus*) pituitary-dependent survival in fresh water reported for teleosts. While we cannot deny the likelihood that phylogenetic differences occur in reliance on the pituitary for freshwater adaptation, present data suggest strongly that the phenomenon is widespread, if not ubiquitous, in freshwater and euryhaline teleosts and that it broadly crosses taxonomic boundaries. We suspect that the mechanism, involving prolactin in every case yet tested, is a primitive feature of teleosts and that differences between taxa in the importance of the mechanism are principally a function of the environmental history of the group.

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Summary

1. Eight species and one subspecies of *Fundulus* native to brackish-water environments fail within 1–10 days following abrupt transfer to fresh water and fail between 1–4‰ when subjected to gradual declines in salinity following hypophysectomy. Intact specimens of most species survive indefinitely in fresh water.

2. Of fourteen species of *Fundulus* and one species of *Profundulus* typical of freshwater habitats, none fail before one month and some survive for periods of up to one year following hypophysectomy and transfer to fresh water.

3. One inland species of *Fundulus* characteristic of saline and freshwater environments demonstrates a failure pattern intermediate between the brackish-water and fresh water species of the genus.

4. In those species tested at two or more different temperatures, low temperature generally prolongs survival in fresh water after hypophysectomy.

5. Although hypophysectomy has only minor effects on serum electrolytes (sodium and chloride) in the three species of *Fundulus* tested in sea water, it

results in marked declines in serum sodium and chloride in fresh water in the five species tested. Extremely low serum electrolytes are associated with failure: the levels at failure do not differ between species but the rate of decline is much slower in freshwater species than in brackish-water forms.

6. Hypophysectomized *F. heteroclitus* fail even at salinities iso-osmotic with serum (12‰), although survival in dilute sea water is much prolonged over that in fresh water and fish survive indefinitely in sea water (29‰). Failure in dilute sea water is not associated with very low serum chloride and sodium, suggesting that factors other than the regulation of these electrolytes may be involved in the process of failure.

7. We conclude that the relative importance of the pituitary in adapting to fresh water is closely correlated with normal environmental salinity in the genus *Fundulus;* the hypophysis being most critical to those species which are subjected to both marine and freshwater conditions in nature.

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